PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 69338-72452	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/SE 03/01499	International filing date *(day/month/year)* 26.09.2003	Priority date *(day/month/year)* 27.09.2002
International Patent Classification (IPC) or both national classification and IPC C12Q1/68		
Applicant BIOTAGE AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15.04.2004	17.02.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Hermann, P Telephone No. +49 89 2399-7109

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/SE 03/01499

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-38	as published

Claims, Numbers

32-35	as published
1-31	received on 21.10.2004 with letter of 15.10.2004

Drawings, Sheets

1/11-11/11	as published

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☒ the claims, Nos.: 32-35

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-31
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	-
	No:	Claims	1-31
Industrial applicability (IA)	Yes:	Claims	1-31
	No:	Claims	-

2. Citations and explanations

   see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

D1: WO-A-0220837
D2: WO-A-0050642
D3: Makeyev E. V. *et al.* - 'Primer-independent RNA sequencing with bacteriophage phi-6 RNA polymerase and chain terminators' - 2001 - *RNA*, **7**: 774-781 (cited by the applicant)
D4: Sasaki N. *et al.* - 'Identification of stable RNA hairpins causing band compression in transcriptional sequencing and their elimination by use of inosine triphosphate' - 1998 - *Gene*, **222**: 17-24
D5: Gerard G. F. *et al.* - "The role of template-primer in protection of reverse transcriptase from thermal inactivation" - 2002 - *Nucl. Acids Res.*, **30**: 3118-3129 (cited by the applicant)

2. The amendments filed with the response to the Written Opinion dated October 15th, 2004, within the prescribed time limit, does not introduce subject-matter which extends beyond the content of the application as filed, and therefore meet the requirements of Article 34(2)(b) PCT.

3. No document at hand discloses the method of present claims 1-31 therefore said claims are novel and meet the requirements of Article 33(2) PCT.

4. However, claims 1-31 do not fulfill the requirements of Article 33(3) PCT the reasons being as follows:

4.1 Document **D1** which discloses a method of typing or identifying one or more nucleic acid (RNA or DNA) molecules (cf. **D1** p. 7 line 20-37; p. 8 line 35 - p. 9 line 25; claim 1), said method comprising the steps of:
- sequentially performing two or more primer extension reactions;
- determining the pattern of nucleotide incorporation thereby providing the typing

information about the variable site on the target molecule (cf. **D1** claim 3); is considered to represent the closest prior art for independent claim 1.

The difference between the method of D1 and that of present claim 1 lies in the fact that the method of independent claim 1 specifies that the hybirdization is performed in the presence of at least one RNase inhibiting agent and that the RNA dependent polymerase lacks RNaseH activity. The effect of such differences lies in the obtention of a safer method avoiding any potential target RNA destruction.

In view of document D1, the problem to be solved by present independent claim 1 can be seen in the obtention of an improved method for the determination of the identity of nucleotide in RNA molecules.

The solution to said problem, i.e. the use of RNAse inhiibitor and of RNA dependent polymerase lacking RNaseH activity, is however considered to represent an obvious option the skilled person would select without the exercise of inventive skills. The person skilled in the art of experimentation with RNA material will be aware of the fragility of said material, and will implement all the requirements for protecting the said molecules from degradation and digestion. At the relevant filing date of the present application RNase inhibitors as well as RNA dependent polymerase lacking RnaseH activity and the advantage of their use, as recited in document **D5** (cf. **D5** p. 3118 right hand-column last § - p. 3119 left.hand column 1st §) are well known in the art. The benefit of their use in the method of D1 when applied with RNA as starting material were therefore forseeable by the skilled person, and in the absence of an indication of a futher unexpected effect linked to the method of independent claim 1, an inventive step cannot be acknowledged. Thus claim 1 does not fulfill the requirements of Article 33(3) PCT.

4.2 The subject-matter of dependent claims 2-26 does not appear to contain any additional features which, in combination with the features of any claim to which said claims refer, meet the requirements of the PCT with respect to inventive step (Article 33(3) PCT) since these additional features appear to be conventional and do not appear to result in any unexpected effect (Article 33(3) PCT).
For instance:

- **D1** and **D2** already mention that the elongation step is repeated (cf: claim 1 of **D1** or **D2**: relevant for claim 2);

- **D1** and **D2** already mention the incorporation of labelled nucleotides which are then detected therefore "recorded" (cf. **D1** p. 22 lines 19-23; **D2** p. 3 lines 19-21: relevant for claims 3, 4, 9 and 26);
- Document **D2** already discloses the use of a detectable moiety which is neutralized, for example by photobleaching or by cleavage, after the detection (cf. **D2** p. 11 line 18 - p. 13 line 5, p. 21 line 9 - p. 25 line 22: relevant for claims 5, 9, and 10);
- Document **D1** already discloses the recording of nucleotide incorporation by detecting the pyrophosphate released using ATP-sulfurylase and luciferase (cf. **D1** p. 22 line 19 - p. 27 line 13: relevant for claims 6-8)
- Document **D2** already discloses the detection of the incorporation of nucleotide by detecting a change in physical properties of the RNA-molecule such as it's ability to uptake intercalating agent (cf. **D2** p. 4 lines 3-15: relevant for claim 11).
- The choice of the appropriate DNA-polymerase or reverse transcriptase is a routine matter for the person skilled in the art as well as the choice of a DNA or RNA primer suitable for RNA sequencing (see also **D1** p. 18 line 13 - p. 20 line 22; **D3** p. 775 right-hand column 2nd § - p. 779 right-hand column 3rd §: relevant for claims 12-13, 18, and 20);
- The choice of the appropriate conditions of temperature, buffer (salt and salt concentration), pH of the buffer, concentration of deoxyribonucleotides or ribonucleotides, or analogs thereof, all constitute routine design procedure which are not suitable to form the basis for the acknowledgement of an inventive step (relevant for claims 14-17, 26);
- When PPi is measured as an indication of nucleotide incorporation, the interference of dATP in the measurement procedure is well known in the art and **D1** already suggests to use S-dATP analog to avoid such interference (cf. **D1** p. 25 line 34 - p. 27 line 13: relevant for claims 19 and 21);
- The interference due to secondary structure in sequencing method is well known and secondary structure reducing agent such as formamide, DMSO, glycerol or SSB are well known by the skilled person (see also **D1** p. 27 line 5-13: relevant for claim 22);
- The advantage of increasing the amount of molecule to be sequenced is well known in the art of sequencing and method for RNA amplification are also known by the skilled person and can not form a basis for inventive step acknowledgment (relevant for claim 23);

- The use of rITP in place of rGTP in RNA sequencing procedure to reduce the base compression is routine in the art (see **D4** abstract and p. 21 left-hand column last § - p. 23 right-hand column 1st §: relevant for claim 27) therefore the modification of the method of **D1** by the replacement of rGTP with rITP arriving therefore to the method of claim 24 can not be considered inventive;
- Sequencing using either immobilized primer or immobilized target nucleic acid is also well known and already suggested in **D1** and **D2** (cf. **D1** p. 31 line 18 - p. 34 line 16; **D2** p. 25 line 25 - p. 26 line 16: relevant for claim 25)

4.3 Document **D1** can be considered as the closest prior art for the method of independent claim 29.
The method of claim 29 differs from the method of independent claim 1 by the only fact that the proportion in the mixture between the nucleotide and the labelled derivative of said nucleotide is given within certain ranges.
Therefore the method of independent claim 29 is distinguished from the method of document **D1** by the features already mentioned as regards the subject-matter of independent claim 1 and the above mentioned additional feature; i.e. the proportion of labelled /unlabelled nucleotide. Said additional feature cannot however be considered to form a basis for inventive step acknowledgement because it is routine in the art of nucleic acid labelling and sequencing that a mixture of cold versus hot nucleotide should be used (as example cf. **D3** p. 780 right-hand column 2nd §; **D4** p. 18 left-hand column 3rd § - p. 19 left-hand column 1st §) and the determination of the proportion between the labelled and non-labelled nucleotide to be used in sequencing procedure, can be determined by the skilled person using conventional and routine experimentation without exercise of inventive skills.
Since the features distinguishing the method of claim 1 from the method of **D1** are not considered inventive (cf. point 3.1 above) and the additional distinguishing feature from claim 29 is not considered inventive either, claim 29 does not fulfill the requirements of Article 33(3) PCT.

4.4 The additional feature brought into the method of claim 29 by dependent claim 30 cannot form a basis for inventive step acknowledgement because, as already mentioned above under point 4.2, removing/impairing the label to be detected by for example photobleaching after the detection step, is well known in the art (Article 33(3) PCT).

4.5 In the light of the objections put forward under points 4.1-4.4 above, for the person skilled in the art, grouping the materials used for a series of known or obvious experiments in the form of a kit is not inventive. Thus, claims 27, 28 and 31 do not meet the requirements of Article 33(3) PCT.

5. Further comments

5.1 Although claims 1 and 29 as well as claims 27 and 31 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought and in respect of the terminology used for the features of that subject-matter. The aforementioned claims therefore lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection (Article 6 PCT).

5.2 Claim 24 lacks clarity (Article 6 PCT) because the exact meaning of the expression "whereby the nucleotide rITP is exchanged for rGTP" is difficult to define and said expression has been interpreted as meaning "wherein the nucleotide rGTP is exchanged by rITP". For instance the correction as not been made.

ART 34 AMDT

Claims (041015):

1. Method for the determination of the identity of at least one nucleotide in a RNA-molecule comprising the steps of:
   (a) providing a single stranded form of the RNA-molecule;
   (b) hybridising an oligonucleotide primer binding to a predetermined position of the RNA molecule, whereby the hybridisation is performed in the presence of at least one RNase-inhibiting agent;
   (c) performing at least one primer extension reaction, whereby the oligonucleotide primer is extended on the RNA-molecule through incorporation of at least one nucleotide by the action of a RNA dependent polymerase, whereby the polymerase is a reverse transcriptase (RT) that essentially lacks RNase H activity;
   (d) detecting the presence or absence of incorporation, thereby indicating the nucleotide identity of the RNA molecule in the relevant position;
   whereby step (c) to (d) optionally are repeated.
2. Method according to claim 1, whereby step (c) to (d) are repeated.
3. Method according to claim 1 or 2, whereby the incorporated nucleotide(s) is (are) recorded.
4. Method according to claim 1-3, whereby the presence or absence of incorporation is indicated by the presence of a detectable moiety.
5. Method according to claim 4, wherein the detectable moiety is removed or neutralized in step (d) after the detection.
6. Method according to claim 1-5, whereby the primer extension reaction results in the release of a residue molecule.
7. Method according to claim 6, whereby the primer extension reaction results in the release of a PPi molecule only upon incorporation of a nucleotide.
8. Method according to claim 7, wherein step (c) is performed by including enzymes, comprising luciferase, apyrase, and ATP-sulfurylase, and reagents to detect the release of PPi to trigger the release of light.

9. Method according to claim 1-8, whereby at least one nucleotide is labelled, such as fluorescently of radioactively, thereby allowing the detection of step (c) to be performed by means of detecting the presence or absence of a labelled nucleotide.
10. Method according to claim 9, whereby the label on the labelled nucleotide is cleavable.
11. Method according to any one of the preceding claims, whereby the detection of step (c) is performed by means of detection of a change in physical properties of the RNA-molecule.
12. Method according to any one of the preceding claims, whereby the RT polymerase is chosen from the group comprising: HIV-1 RT, M-MuLV RT, AMV RT, RAV2 RT, Thermoscript AMV RT, Superscript II M-MuLV RT, Tth DNA polymerase, Superscript II RNase H$^-$ RT.
13. Method according to any one of the preceding claims, whereby a mixture of RNA dependent polymerases is added to the reaction mixture of step (a).
14. Method according to any one of the preceding claims, whereby the extension reaction is performed at a temperature ranging from 28 to 70 °C.
15. Method according to any one of the preceding claims, whereby the pH of the extension reaction solution is in the interval from 7.6 to 8.6, preferably from 8.0 to 8.4.
16. Method according to any one of the preceding claims, whereby the concentration of deoxynucleotides is in the interval from 1 µM to 1 mM.
17. Method according to any one of the preceding claims, whereby the salt concentration of the reaction mixture is in the interval from 10 to 100 mM.
18. Method according to any one of the preceding claims, wherein the oligonucleotide primer is a DNA primer.
19. Method according to claim 18, whereby the nucleotide is the deoxynucleotide dATP, which further is exchanged for the analogue alpha-S-dATP.
20. Method according to claim 1-18, wherein the oligonucleotide primer is a RNA primer.
21. Method according to claim 20, whereby the nucleotide ATP is exchanged for the analogue alpha-S-ATP

22. Method according to any one of the preceding claims, whereby a RNA-secondary structure reducing reagent, preferably chosen from the group comprising T4 Gene 32 Protein, retroviral nucleocapsid protein, actinomycin D, glycerol, methyl mercury hydroxide, methoxyamine-bisulfite, DMSO, spermidine, formamide, SSB (single stranded binding protein) and blocking primer, is included in the extension reaction.
23. Method according to any one of the preceding claims, whereby the RNA molecule is subjected to an RNA amplification prior to the extension reaction.
24. Method according to claim 23, whereby the nucleotide rITP is exchanged for rGTP in the amplification.
25. Method according to any one of the preceding claims, wherein the oligonucleotide primer is immobilised to a solid phase or wherein the RNA molecule is captured to a solid phase by an immobilised oligonucleotide.
26. Method according to any one of the preceding claims, whereby the quantity of the RNA-molecule is determined by measuring the intensity of the incorporation signal and comparing it to a reference.
27. Kit for performing the nucleotide identification of claim 1-26, comprising in separate vials a reverse transcriptase that essentially lacks RNase H activity, nucleotides, necessary enzymes for a sequencing-by-synthesis reaction, and optionally other necessary reagents.
28. Kit according to claim 27, which further comprises a RNA quantity reference sample.
29. Method for determining the sequence of a ribonucleic acid molecule comprising the steps of;
    a) providing a single-stranded form of said ribonucleic acid molecule;
    b) hybridizing a primer to said single stranded form of said ribonucleic acid molecule to form a template/primer complex, whereby the hybridisation is performed in the presence of at least one RNase-inhibiting agent;
    c) enzymatically extending the primer by the addition of an RNA dependent polymerase and a mixture of nucleotides and a derivative of said nucleotides, wherein the derivative of said nucleotide comprises a label linked to a

ART 34 AMDT

nucleotide via an optionally cleavable link and wherein the proportion in the mixture between the nucleotides and the derivative of said nucleotide is within the range of 1-60%, 1-50%, 1-40%, 1-30%, or 1-20%, preferably in the range of 5-60%, 5-50%, 5-40%, 5-30%, or 5-20%, or more preferably in the range of 10-60%, 10-50%, 10-40%, 10-30%, or 10-20%, whereby the polymerase is a reverse transcriptase that essentially lacks RNase H activity;

d) determining the type of nucleotide added to the primer;

30. Method according to claim 29, wherein the label is neutralized after step d) by the addition of a label-interacting agent or by bleaching, preferably by photo-bleaching.

31. Kit comprising, in separate compartments, a mixture of natural nucleotides and a derivative of said nucleotides according to step c) of claim 29, and at least one of the following components: a reverse transcriptase that essentially lacks RNase H activity, a reducing agent, a carrier, a capping agent, an apyrase, an alkaline phosphatase, a PP-ase, a single strand binding protein or the protein of Gene 32, for performing the method according to claim 29-30.